(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) Publication number: **0 190 050 B1**

(12) EUROPEAN PATENT SPECIFICATION

(45) Date of publication of patent specification: **15.05.91** (51) Int. Cl.⁵: **A61K 9/133**, B01J 13/02

(21) Application number: **86300641.7**

(22) Date of filing: **30.01.86**

(54) Method for preparing small vesicles using microemulsification.

(30) Priority: **31.01.85 US 696727**

(43) Date of publication of application:
06.08.86 Bulletin 86/32

(45) Publication of the grant of the patent:
15.05.91 Bulletin 91/20

(84) Designated Contracting States:
AT BE CH DE FR GB IT LI LU NL SE

(56) References cited:
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EP-A- 0 107 559
US-A- 4 310 506

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EP 0 190 050 B1

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Description

FIELD OF THE INVENTION

5 This invention relates to a process for producing small lipid micellular particles in the form of unilamellar vesicles in commercial quantities by microemulsifying lipid compositions using very high shear forces.

10 BACKGROUND OF THE INVENTION

Unilamellar phospholipid micellular particles in the form of vesicles (also known as liposomes) have received increasing attention from researchers as carriers of various substances, such as imaging agents and for diagnosis of abnormalities such as tumors in humans using animal models. In particular, it has been 15 shown that small vesicles (less than 200 nm (2000 Å)) may be labelled to target tumors (Proffitt, et al., J. Nucl. Med. 24(1), p. 45-50 (1983)).

Such vesicles are also useful as potential carriers of therapeutic agents for treatment of tumors. Alternatively, small vesicles are useful for in vitro immunoassays, U.S. Pat. No. 4,342,826 and D. Papahadjopoulos (Ed.) Annals N.Y. Acad. Sci., 308 (1978). Additionally, the vesicles containing imaging or 20 therapeutic agents may be modified by incorporating various carbohydrate derivatives into the vesicle surface to increase tissue specificity of the vesicles, or by adding cholesterol to increase the stability of the vesicles. Mauk and Gamble, Anal. Bioc. 94, pg. 302-307 (1979); Mauk, et al., P.N.A.S. (U.S.A.) 77(8), pg. 4430-4434 (1980); and Liposome Technology, Targeted Drug Delivery and Biological Interaction, Vol. III, G. Gregoriadis (Ed.), C.R.C. press, Inc. (1984).

25 The prior art shows that vesicles such as liposomes may be produced using the methods of sonication, dialysis, injection or reverse phase evaporation. These procedures are well known and may be found in the following articles: Huang, Biochemistry 8, pg. 344 (1969) (Sonication); Rhoden and Goldin, Biochemistry 18, pg. 4173 (1979) (dialysis); and Kremer et al Biochemistry 16, pg. 3932-3935 (1977) (injection); and Liposome Technology, Preparation of Liposomes, Vol. I, 6 Gregoriadis (Ed.), CRC Press Inc. (1984). These 30 methods share several disadvantages including the inability to conveniently produce commercial quantities of such vesicles.

The use of homogenizing devices to produce emulsions from solutions with soluble and insoluble components is well known in the art, U.S. Pat. No. 4,127,332. Several such homogenizing devices operate by creating shearing forces to disperse the insoluble and soluble components. These shearing forces result 35 from the process known as cavitation which involves the rapid formation of bubbles within the sample solution as it passes through narrow channels causing a reduction in the vapor pressure of the fluid. The bubbles then collapse as the solution moves out from these channel areas, generating a shearing force. Such homogenizing devices, however, have been operated at relatively low pressures (usually below 69 MPa (10,000 psi) for the purpose of creating emulsions with large particles (greater than µm 1) such as 40 lipoproteins for baking purposes, (U.S. Pat. No. 4,360,537), or simply to form an emulsion of oil and water, U.S. Pat. No. 4,026,817.

Recently, various mechanical devices such as homogenizers have been employed in producing vesicles, U.S. Pat. No. 4,411,894. However, these devices have been used to assist with the initial dispersion of vesicle precursor substances such as soya or egg lecithin which do not require high shear 45 forces to form vesicles and which do not form vesicles optimally stable in vivo. In addition, the French Press and Pressure Cell has been used to generate small vesicles, U.S. Patent No. 4,263,428. A disadvantage of this device is that it requires extra time to reload a sample since it provides no means to recirculate the lipid solution through the device.

It is, therefore, an object of the present invention to provide an efficient, time-saving and reproducible 50 process, having the advantages enumerated above for producing commercial quantities of small, unilamellar vesicles, especially vesicles suitable for treatment and diagnosis of tumors in a body.

SUMMARY OF THE INVENTION

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The present invention comprises a process for the production of small (less than 200 nm (2000Å)) unilamellar vesicles in commercial quantities wherein a solution containing lipids and other components capable of forming the desired vesicles is placed in a modified homogenizing apparatus, maintained at a

selected temperature, and subjected therein to very high shearing forces, for a selected time.

The process of this invention further comprises a method for preparing small (less than 200 nm (2000 Å))unilamellar lipid vesicles suitable for use as carriers of imaging agents for targeting tumor cells in a body. These vesicles are prepared by placing a solution of components capable of forming vesicles, an ionophore, a chelating agent and, in some applications, a radioactive tracer bound to said chelating agent, in a homogenizing apparatus and subjecting the solution to very high shearing forces while maintaining the solution at a selected temperature, for a selected time.

This invention also includes a method for preparing small (less than 200μm (2000Å)) unilamellar vesicles suitable for use as carriers of therapeutic agents for treating tumors in a body. These vesicles are obtained by placing a solution of components capable of forming vesicles, a therapeutic agent and, in some applications, an ionophore, a chelating agent and a radioactive tracer bound to said chelating agent, in a homogenizing apparatus and subjecting this solution to very high shearing forces while maintaining the solution at a selected temperature, for a selected time.

The present invention provides a process for the preparation of small, unilamellar vesicles of diameter less than 2000 Å (200 nm) suitable for biological applications comprising:

(a) hydrating amphiphilic molecules which are phospholipids or phosphoglycerides having hydrocarbon chains of at least 16 carbon atoms, and optionally other components capable of forming lipid vesicles that are stable in vivo;

(b) dispersing said hydrated lipid in a homogenizing apparatus having recirculation means at a pressure of 55 to 90 MPa (8000 to 13000 p.s.i.) and at a temperature of 50°C to 80°C to generate a microemulsion containing small unilamellar lipid vesicles of less than 200 nm (2000 Å); and

(c) separating said small, unilamellar vesicles from any unencapsulated materials.

DESCRIPTION OF THE PREFERRED EMBODIMENT

The process of the present invention begins with the preparation of a solution of materials capable of forming vesicles. Preferably the lipids for use in the present invention are phospholipids and may include dipalmitoyl phosphatidyl-choline (DPPC), distearoyl phosphatidylcholine (DSPC) or similar materials.

Amphiphilic molecules other than phospholipids such as phosphoglycerides may also be used. See generally, The Hydrophobic Effect by Charles Tanford, Wiley-Interscience, (1980), Biological Lipids, (Ch. 11) pg. 106-109. It is preferable to use compounds with hydrocarbon chains which exhibit phase transitions at relatively high temperatures (greater than 37°C) to form vesicles with improved stability in vivo. It is known that phase transition points are a function of hydrocarbon chain lenth, The Hydrophobic Effect, Charles Tanford, (2nd Ed. 1980). Thus, vesicle-forming compounds with carbon chains of at least 16 carbon atoms are preferable. However, it is more difficult to accomplish vesicle formation with such longer hydrocarbon chains.

I have surprisingly found that the use of a homogenizing apparatus operated at higher pressures then specified, and equipped with a reservoir capable of maintaining selected temperatures can convert such long-chain hydrocarbon compounds into improved vesicles which can thus be produced in commercial quantities.

Cholesterol may be incorporated into the lipid solution to increase the stability of the vesicles which are prepared using the process disclosed herein.. In addition, if the vesicles are used to carry imaging agents for locating and diagnosing tumors, a chelating compound may be added to the lipid solution to become entrapped within the vesicles, as well as an ionophore for loading external cations for radiolabelling into the chelating agent within the vesicles. Imaging is accomplished using a gamma-camera. The preferred ionophore is A23187, but other useful ionophores are polyethers such lasalocid A(X-537A) and 5-Bromo derivatives of lasolocid; cyclic depsipeptides such as beauvericin; cyclic peptides such as valinomylin; and anti-fungal toxins such as arenaciolide. The chelating agent is preferably nitriloacetic acid (NTA), although other chelators may also be used. For example, where the cations are polyvalent metal ions, polyamino carboxylic acid chelators for such ions may be employed, such as ethylenediamine-tetracetic acid, diethylene-triamine-pentaacetic acid, diamino-cyclohexanetetra-acetic acid and iminodiacetic acid. Other agents useful for imaging tumors may include contrast agents for X-ray imaging such as diatrizoic salts, for example Hypaque® meglumine, or Nuclear Magnetic Resonance (NMR) imaging agents such as paramagnetic ions and their complexes with strong chelating agents, for example, Gadolinium-DTPA.

This invention also contemplates the use of vesicles to carry therapeutic agents to treat abnormalities such as tumors in a patient. Chemotherapeutics such as antibiotics (including Daunomycin, Bleomycin, Adriamycin, Actinomycin D, Mytomycin C or Mithramycin), alkylating agents (including chlorambucil,

cyclophosphamide or Triethylenemelamine) or antimetabolites (including methotrexate, 5-Flourouracil, 6-Mercaptopurine, Arabinosyladenine or Arabinosylcytosine may be attached to the vesicles during the microencapsulation process of this invention. Alternatively, radiotherapeutic agents including radionuclides such as Iodine 131, Yttrium 90 or Phosphorus 32 may be attached to the vesicles produced, using the methods disclosed herein, for radiotherapy of tumors.

This invention requires the use of a homogenizing apparatus capable of operation at high pressures to generate the very high localized shearing forces necessary to produce the microemulsion of small, unilamellar lipid vesicles from the solutions of vesicle-forming materials disclosed herein. Such an apparatus is a modified Gaulin Homogenizer (Model 15M) which accomplishes dispersion of the lipid solution by means of a homogenizing valve. The specification for the homogenizer prescribes a continuous operating pressure of 55 MPa (8,000 p.s.i.) or an intermittent operating pressure of 69 MPa (10,000 p.s.i.). I have found, however, that with proper safety precautions the homogenizer can operate for short periods of time for up to 83 MPa (12000 p.s.i.) or even 90 MPa (13000 p.s.i of pressure. In the preferred embodiment, the homogenizer recirculates solution past the homogenizing valve at the rate of 1 liter per minute.

The Gaulin homogenizer is modified with two heat exchange reservoirs maintained at 5-10° C and 80° C and equipped with a feedback loop to assist in converting the longer chain hydrocarbon vesicle-forming components into vesicles. When higher temperatures are used in the heat exchange reservoir, a wider range of pressure settings may be used to generate vesicles of the desired size. Since the actual temperature of the lipid solution is several degrees higher in the homogenizing valve area where the shearing forces are generated, at the higher reservoir temperatures higher pressures may not be advisable because they may further increase the temperature of the solution in the dispersing valve area. The higher temperature of operation thus allows one to lower the pressure settings and still generate vesicles of suitable dimensions for various biological applications. As noted above, this effect is probably due to the increasing ease of converting larger chain hydrocarbon amphiphilic molecules into vesicles at temperatures above their phase transition points.

This invention also makes use of a Nicomp 200 Laser Particle Size Instrument which determines the distribution of particle sizes in a solution using the principles of dynamic light scattering. Briefly, laser light is passed through a vesicle sample solution and particle size is determined from the time behavior of fluctuations in the scattered light intensity using a series of time-dependent calculations expressed as an auto-correlation function. Particle hydro-dynamic radius (Rh) is calculated by the Stokes-Einstein relation using least squares analysis. The mean radius and variation of the particle distribution produced by the Nicomp from a sample is obtained by assuming that the distribution is Gaussian in shape. However, when there is a bimodal particle size distribution this assumption is not appropriate and the manufacturers of the Nicomp have provided proprietary instrument programs which enable the sample data to be assigned to a bimodal distribution to obtain the average mean diameter (a function of Rh) for the particles in such a distribution. A bimodal size distribution was obtained for vesicles prepared by the methods of the present invention. Using the data fitting program purchased from the Nicomp Manufacturers (Santa Barbara, CA), values for the average mean diameter (Stokes-Einstein (Rh x 2)) of the vesicles in a sample were obtained. In addition, microscopic examination of vesicles in several samples run through the Nicomp Size Instrument revealed that the diameters of a majority of vesicles actually fell within the main peak of the bimodal size distribution curve obtained from the Nicomp. Thus, for the size data reported in the examples herein, the diameters of the vesicles in a given sample prepared by the methods of this invention are given as both the average Stokes-Einstein and the average main peak vesicle diameters.

For a more detailed explanation of dynamic light scattering, see B. Chu, Laser Light Scattering, Academic Press, N.Y. (1974), and see instructional materials accompanying the Nicomp Size Instrument.

BRIEF DESCRIPTION OF THE DRAWINGS

The following examples are presented to illustrate the invention. In the examples, reference is made to FIGS. 1, 2 and 3 of the drawings and Tables I-III.

FIG. 1 is a front view of a modified Gaulin homogenizing apparatus.

FIG. 2 is a side view of a modified Gaulin homogenizing apparatus.

FIG. 3 is a cross-section of the homogenizing valve assembly taken along line 3-3 of FIG. 2.

EXAMPLE I

PREPARATION OF VESICLE SOLUTION

Vesicle solutions were prepared using the techniques described by Mauk et al., Anal Bioc. 94 pg. 302-307 (1979), and disclosed in U.S. Patent No. 4,310,506. Briefly, L- α-distearoyl phosphatidylcholine (DSPC) from Calbiochem, was used as the phospholipid component of the vesicle solution without further purification. Cholesterol (CH) was purchased from Sigma, and the trisodium salt of nitriloacetic acid (NTA) from Aldrich Chemical Co. The ionophore, A23187 was obtained from Eli Lilly and Co.; its preparation is described in U.S. Patent No. 3,960,667.

For this Example, DSPC and cholesterol were used in a mole ratio of 2:1 (5 g total lipid, DSPC and CH), and dissolved in 50 ml of chloroform, then dried to a film in a rotary evaporator. The film was dried under vacuum overnight and rehydrated with 0.5 liter phosphate buffered saline (P_i/NaCl: 0.9% NaCl/5mM sodium phosphate at a pH of 7.4). The concentration of total lipid was approximately 10 mg/ml total lipid.

A modified Gaulin Homogenizer, Model 15M, as shown in FIGS. 1 and 2 was used to carry out microemulsification of the lipid solution. The homogenizer consists of a transmission, 4, a pressure adjusting screw, 5, a pressure gauge, 6, a recirculation loop 7, and a homogenizing valve assembly, 8. The modification consists of two heat exchange reservoirs, 9 and 10 in FIG. 2 which maintain the lipid solution at a selected temperature in the range of 40°-80°C, depending on the pressure at which the sample will be run, but preferably between 70°-75°C. One reservoir, 9 of FIG. 2 is kept in the range of 5°-10°C, the other reservoir, 10 of FIG. 2 is kept at approximately 80°C.

In operation the lipid solution prepared as above is placed in the solution receptacle, 11 of FIG. 1, and is then moved into the valve area 12 of FIG. 3 at high pressure and low velocity. Vapor bubbles form in the solution as a result of the rapid increase in velocity accompanied by a decrease in pressure as the solution moves through the channel 13 between the valve 14 and valve seat 15. The vapor bubbles then implode as the solution exits the valve area 12 at a lowered velocity and at an increased pressure. This process of bubble formation and implosion (also known as cavitation) generates the high shearing forces which microemulsify the lipid solution. The microemulsion then exits the valve area 12, impinges on the impact ring 16 and recirculates through the homogenizer.

It is preferable that the homogenizing apparatus is operated for a time sufficient to allow a number of circulations of the entire lipid solution through the homogenizing valve area (12 of FIG. 3) to achieve optimal micromulsification. Taking into consideration the volume (0.5 liter) and flow rate (one liter/min.) of the modified Gaulin homogenizing apparatus used in this Example, at least 20 circulations (corresponding to approximately 10 minutes) but not greater than 200 circulations (100 minutes) were found to be sufficient to produce a microemulsion of small vesicles suitable for biological applications.

In this Example, vesicle compositions were run through the homogenizer at temperatures selected in the range of 50°-80°C for time periods ranging from 15-90 minutes. The pressure for each run varied between 55 MPa to 83 MPa (8000 p.s.i. to 12000 p.s.i.).

After each run, the size of the vesicles in the microemulsion was determined.

VESICLE SIZING

Approximately 1 milliliter of the homogenized vesicle suspension was centrifuged at 15,000 rpm for 10 minutes using an Eppendorf Microcentrifuge Model 5414. Large particles which would cause error in the light scattering measurement are pulled to the bottom while the vesicles remain suspended. A 6 x 50mm test tube was rinsed with filtered PBS then filled to within 5 mm of the top with PBS. 3-4 μl of the centrifuged vesicles were then placed in the test tube and the contents mixed by inverting the tube several times. A Nicomp 200 Laser Particle Sizer Instrument was used to determine the average mean diameter and main peak diameter as described above for a sample of vesicles. Use of the Nicomp Particle Sizer is described in the instruction manual. The temperature was set at 20°C. An appropriate channel width (preferably 1.4El m sec) and prescale factor (preferably 1) were selected. The sample was then run through the Nicomp. After 50,000 counts a reasonable estimate of particle size was obtained.

Table I shows vesicle size data summarized for vesicles prepared by the micromulsification procedures described in this example at different pressures for different time periods and at reservoir temperatures ranging from 50°-80°C.

TABLE I

TEMP (°C)	PRESSURE MPa (PSI)	*AVERAGE MAIN PEAK DIAMETER OF VESICLES	SIZE (nm) AVERAGE STOKES-EINSTEIN DIAMETER	HOMOGENIZING TIME (MIN)
50-55	76(11000)	67	102	15
50-55	76(11000)	61	99	30
50-55	76(11000)	57	100	60
50-55	76(11000)	57	106	75
50-55	76(11000)	56	111	90
50-55	83(12000)	59	97	15
50-55	83(12000)	55	94	30
50-55	83(12000)	51	90	60
50-55	83(12000)	47	87	75
70-75	76(11000)	63	122	15
70-75	76(11000)	57	116	30
70-75	76(11000)	52	107	60
70-75	76(11000)	50	83	75
70-75	76(11000)	51	88	90
70-75	55(8000)	57	103	15
70-75	55(8000)	59	100	30
70-75	55(8000)	54	89	60
70-75	55(8000)	52	88	75
70-75	55(8000)	52	84	90

The data in Table I shows that small lipid vesicles (less than 200 nm (2000 Å))are obtained by microemulsification using high shear forces generated in a homogenizer operated at high pressure.

At the lower reservoir temperature 50°-55°C) small vesicles are reproducibly generated at higher pressures (greater than 69 MPa (10,000 psi). Vesicles are preferably obtained by microemulsification at the higher reservoir temperatures (70-75°C) which generate suitable small vesicles at pressures greater than 55 MPa (8,000 psi). This high temperature effect thus allows a greater range of pressures to be used which may be a function of the difficulty of converting longer-chain hydrocarbons into vesicles due to higher phase transition points. A reduced temperature necessitates operation of the homogenizing apparatus at higher pressures to generate sufficient shearing forces to convert such vesicle precursors into vesicles.

Such vesicles are useful for various biological applications, such as diagnosis and treatment of tumors and in vitro assays.

5 EXAMPLE II

PREPARATION OF VESICLES MODIFIED FOR IMAGING TUMORS

10 Vesicle solutions were prepared as in Example I with the following modifications: the ionophore A23187 was added to the DSPC:CH mixture, to yield a mole ratio for DSPC,CH,A23187 of 2:1:0.004 (5 g total lipid, DSPC and CH) using the procedures disclosed in U.S. Patent Nos. 4,310,506, 3,960,667 and in Mauk et al., P.N.A.S. U.S.A., 76, (2) 765-769 (1979). A23187 permits loading of lipid vesicles with a radiolabelling cation such as $^{111}In^{3}$ The inclusion of small amounts of A23187 does not interfere with the formation of unilamellar 15 vesicles by the microemulsification procedure.

The DSPC, CH and A23187 components were dissolved in 50 ml chloroform and dried to a thin lipid film as above. The dried lipid film was then rehydrated with a 0.5 liter PBS solution containing the weak chelator NTA (1mM), at pH 7.4. The concentration of total lipid was approximately 25 mg/ml. As disclosed in U.S. Patent No. 4,310,506 and Mauk, et al., P.N.A.S. USA 76 (2), pg. 765-769 (1979), NTA provides the 20 driving force for the net transfer of cations for radiolabelling into the vesicles. While NTA is the preferred chelator as mentioned above, other chelators may be used. In addition, while $^{111}In^{3}$ is the preferred cation for radiolabelling vesicles for biodistribution studies and diagnostic procedures, any cation which can be bound to a chelating agent may be used. The cations are preferably selected from the group of radioactive tracers, for example ^{111}In, ^{45}Ca, ^{51}Cr, ^{99}Tc, ^{67}Ga, ^{57}Co and ^{65}Zn.

25 After rehydration with the NTA in PBS, the mixture was microemulsified in a modified Gaulin Homogenizer, as described in Example I. A range of time periods and pressure settings were used as described in Example I. The preferred parameters for producing small vesicles suitable for the biodistributions of this example were found to be microemulsification for 60 minutes at 69 MPa (10,000 p.s.i.) with a solution temperature of 70° C.

30 The microemulsion obtained was then filtered by standard gel filtration techniques to separate larger particulate matter and excess (unencapsulated) NTA from the small vesicles encapsulating NTA. The small vesicles were then concentrated using an Amicon Hollow-Fiber concentrator apparatus, and the total lipid concentration determined using a phosphate assay. PBS was then used to dilute the vesicles to a final total lipid concentration of 25 mg/ml.

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LOADING

Vesicles were then loaded with $^{111}In^{3}$ using the procedures described by Mauk and Gamble, Anal. Bioc. 40 94, pg. 302-307 (1979). Briefly, 500 μL (5 mg lipid) of vesicles were incubated with 35 μl of 3.4 μM $InCl_3$ in 104 mM sodium citrate, (pH 7.4) and 1-50 μl of $^{111}In^{3}$ depending on the required activity. A volume of 2 x PBS equal to that of the $^{111}In^{3}$ addition was included in the incubation mixture. Maximal loading was accomplished by incubating at 80° C.

The vesicles were analyzed to determine suitability for biodistribution and targeting studies as 45 compared with vesicles obtained by sonication. Sonicated vesicles were prepared as described by Mauk and Gamble, Anal. Bioc. 94, pg 302-307 (1979) and U.S. Pat. No. 4,310,506. Briefly, a lipid solution of the same composition as discussed above for Example I was dried then rehydrated with 0.5 ml of 1 millimolar NTA in PBS. The mixture was sonicated in a water bath at room temperature for 10 minutes, then incubated at 60° C for 10 minutes to anneal any structural defects. The vesicles were then centrifuged at low speed to 50 remove titanium and any highly aggregated materials. The NTA that did not incorporate was removed by passing the preparation over a Sephadex® G-50 column equilibrated with PBS. The vesicles were then characterized as described below.

55 VESICLE SIZING

Sizing of the vesicles produced by the methods of this Example was accomplished as described for Example I using the Nicomp Sizing Instrument and was compared to results obtained in producing

sonicated vesicles. As shown in TABLE II the methods of this invention yield vesicles with sizes in the range of 40-100nm (400 Å -1000 Å) comparable to sizes of sonicated vesicles, and found by the inventor to be suitable for use in biodistribution and targeting studies.

Table II

Characteristics of Microemulsified Vesicles vs. Sonicated Vesicles

	SIZE (nm)		$^{111}In^{3+}$ Loading Efficiency
	Stokes-Einstein Diameter (Rh x 2)	Main Peak Diameter	
1. Microemulsified	87	66	80.1%
2. Sonicated	83	51	83.1%

LOADING EFFICIENCY

The efficiency of loading the $^{111}In^3$ into vesicles was determined as follows: 100 μL of $^{111}In^3$ vesicle preparation loaded as described above was added to 0.5.g of moistened Chelex® (Dow Chemical Corp.) previously adjusted to pH 7.4 and mixed for two minutes. 900 μL of PBS was added, and the mixture was centrifuged in a tabletop centrifuge for 5 minutes at room temperature. 500 μL of supernatant was removed and the loading efficiency (percentage of vesicles loaded) was determined by counting in a Gamma counter the radioactivity of the 500 μL sample divided by the radioactivity of the 100 μL of original vesicle preparation and multiplying by 200.

As indicated in Table II, the efficiency of loading the cation $^{111}In^3$ into the vesicles produced by microemulsification was found to be greater than 80%, which is comparable to values previously obtained for $^{111}In^3$ -loaded vesicles prepared by sonication. Mauk and Gamble, Anal. Bioc. 94, pg. 302-307 (1979).

BIODISTRIBUTION

To explore the suitability of the vesicles obtained in this example for use in vivo, vesicles containing the radioactive tracer $^{111}In^3$ were administered to BALB/C female mice which were previously implanted subcutaneously in the right thigh with EMT 6 tumors 9-10 days prior to the initiation of these experiments to permit analysis of the biodistribution of the vesicles in animal tissue.

Intravenous injection was made via a lateral tail vein. Each mouse was then weighed and housed for 24 hours. Prior to sacrificing, the mice were anesthetized with ether and 1/2 to 1 milliliter of blood was removed via the orbit and placed into a Beckman Gamma Counter 5500 gamma counting tube. The mice were then sacrificed by cervical dislocation and the following samples dissected: tumor, lung, liver, spleen, kidney, muscle, intestine, stomach, skin, tail and carcass. These samples (excluding muscle, intestine, stomach, skin, tail and carcass), were thoroughly rinsed in PBS and placed in gamma counting tubes and weighed. All samples were counted in the gamma counter for 1 minute to calculate the percentage of injected dose (radioactivity) per gram for each tissue. Two standards of vesicles were counted along with the tissue samples.

The results of biodistribution of radiolabelled vesicles prepared by microemulsification at 69 MPa (10,000 psi) (at temperatures of 70-75 °C) are summarized in TABLE III. Table III also compares these results with data obtained for the biodistribution of vesicles prepared by sonication and labelled with $^{111}In^3$.

TABLE III

BIOLOGICAL DISTRIBUTION OF VESICLES

Average % Injected dose/g Tissue for 5 mice

TISSUE	MICROEMULSIFIED VESICLES	SONICATED VESICLES
1. Blood	8.1	10.4
2. Tumor	34.0	34.0
3. Lung	5.3	6.2
4. Liver	19.8	18.4
5. Spleen	26.2	25.1
6. Kidney	10.6	9.2
7. Muscle	1.2	0.8
8. Intestine	4.1	3.1
9. Stomach	2.3	2.5
10. Skin	3.3	3.7
11. Tail	2.6	2.0
12. Carcass 1-3	1.7	1.5

Tissue distributions were thus found to be comparable for the microemulsified and sonicated vesicles.

These results demonstrate that vesicles produced by the microemulsion process disclosed herein possess attributes of size and stability comparable to vesicles produced by sonication, and are suitable for use in vivo to target tumors for diagnosis and treatment, as well as for other biological applications such as in vitro bio-assays.

Claims

1. A process for the preparation of small, unilamellar vesicles of diameter less than 200 nm (2000 Å) suitable for biological applications comprising:
   (a) hydrating amphiphilic molecules which are phospholipids or phosphoglycerides having hydrocarbon chains of at least 16 carbon atoms, and optionally other components capable of forming lipid vesicles that are stable in vivo;
   (b) dispersing said hydrated lipid in a homogenizing apparatus having recirculation means at a pressure of 55 to 90 MPa (8000 to 13000 p.s.i.) and at a temperature of 50°C to 80°C to generate a microemulsion containing small unilamellar lipid vesicles of less than 200 nm (2000 Å); and
   (c) separating said small, unilamellar vesicles from any unencapsulated materials.

2. A process according to claim 1 wherein the amphiphilic molecules and other components capable of forming lipid vesicles comprise a phospholipid and cholesterol in a mole ratio of 2 to 1.

3. A process according to claim 1 or 2 wherein the final concentration of total lipid in the microemulsion is 10 mg/ml.

4. A process according to claim 1 wherein the step (a) includes:

(a) (i) dissolving said amphiphilic molecules and other components capable of forming lipid vesicles in an organic solvent solution;
(a) (ii) drying said solution to a lipid film; and
(a) (iii) rehydrating the dried lipid film.

5. A process according to claim 4 for the preparation of vesicles suitable for use in targeting tumors in a body for location and diagnosis of the tumors comprising:
in step (a) (i), dissolving the amphiphilic molecules and at least one other component, including an ionophore;
in step (a) (iii), rehydrating the dried lipid film with phosphate bufferred saline containing a weak chelating agent;
in step (b), dispersing said rehydrated lipid to generate a microemulsion containing lipid vesicles with the ionophore incorporated into the lipid bilayer and said vesicles containing the chelating agent;
and, following step (c),
(d) loading said vesicles with a radioactive cation for detecting the location of said vesicles when administered into a body.

6. A process according to claim 4 for the preparation of vesicles for use in treating a tumor in a body comprising:
in step (a) (iii), rehydrating the dried lipid film with a solution containing a therapeutic agent; and
in step (b) dispersing said hydrated lipid to generate a microemulsion containing lipid vesicles containing the therapeutic agent.

7. A process according to claim 4, 5 or 6 wherein said organic solvent is an ether, chloroform or alcohol.

8. A process according to any one of the preceding claims wherein said dispersing step is performed in a homogenizing apparatus in which said lipid solution may be maintained in a selected temperature range.

9. A process according to claim 8 wherein said apparatus comprises a heat exchange reservoir.

10. A process according to any one of the preceding claims wherein said step of dispersing is performed by operating said homogenizing apparatus at a pressure of 55 to 83 MPa (8000 to 12000 p.s.i.) and a solution temperature maintained at 50° to 80° C for a time of from 15 to 90 minutes.

11. A process according to any one of claims 1 to 9 wherein said step of dispersing is performed by operating said homogenizing apparatus at a pressure of 55 to 69 MPa (8000 to 10,000 p.s.i.) and at a reservoir temperature of 70°-75° C for approximately 60 minutes.

12. A process according to any one of claims 1 to 9 wherein said step of dispersing is performed by operating the homogenizing apparatus at a pressure of 69 to 83 MPa (10,000 to 12,000 p.s.i.) and at a reservoir temperature of 50°-55° C for approximately 60 minutes.

13. A process according to any one of claims 1 to 9 wherein said step of dispersing is performed by operating said homogenizing apparatus at a pressure of approximately 69 MPa (10,000 p.s.i.) and at a reservoir temperature of 70°-75° C for approximately 60 minutes.

14. A process according to claim 1 or 6 wherein said amphiphilic molecules are phospholipids which are phosphatidylcholine, phosphatidylethanolamine or phosphatidylserine.

15. A process according to claim 1 or 5 wherein the phospholipid is distearoyl phosphatidylcholine and/or dipalmitoyl phosphatidylcholine.

16. A process according to claim 15 when appendant to claim 5 wherein said amphiphilic molecules are phospholipids and said other components capable of forming vesicles comprise cholesterol and an ionophore.

17. A process according to claim 16 wherein the composition of the vesicles comprises phospholipid, cholesterol and ionophore in the mole ratio 1:2:0.004.

18. A process according to claim 16 or 17 wherein the ionophore is A23187.

19. A process according to any one of claims 5, 16, 17 or 18 wherein the chelating agent is nitriloacetic acid.

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20. A process according to any one of claims 5, 16, 17, 18 or 19 wherein the radioactive cation is $^{111}In^{3}$.

21. A process according to any one of claims 5 or 16 to 20 wherein the final concentration of total lipid in the microemulsion is 25 mg/ml.

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22. A process according to claim 6 wherein the phospholipid is distearoyl phosphatidylcholine.

23. A process according to claim 6 or 22 wherein said step of dissolving lipid substances in an organic solvent further includes the addition of cholesterol.

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24. A process according to any one of claims 6, 22 or 23 wherein the composition of vesicles comprises phospholipid, cholesterol and a therapeutic agent, said therapeutic agent being contained in the solution used to hydrate the amphiphilic molecules.

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25. A process according to any one of claims 6 or 22 to 24 wherein said therapeutic agent is a chemotherapeutic agent.

26. A process according to claim 25 wherein said chemotherapeutic agent is an antibiotic which comprises Daunomycin, Bleomycin, Adriamycin, Actinomycin D, Mytomycin C or Mithramycin.

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27. A process according to claim 25 where said chemotherapeutic agent is an alkylating agent comprising chlorambucil, cyclophosphamide or Triethylenemelamine.

28. A process according to claim 25 wherein said chemotherapeutic agent is an antimetabolite comprising methotrexate, 5-Fluorouracil, 6-Mercaptopurine or Arabinosylcytosine.

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29. A process according to claim 25 wherein said chemotherapeutic agent is methotrexate.

30. A process according to any one of claims 6 or 22 to 24 wherein said therapeutic agent is a radiotherapeutic agent.

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31. A process according to claim 30 wherein said radiotherapeutic agent is a radionuclide which comprises Iodine 131, Yttrium 90 or Phosphorus 32.

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Revendications

1. Procédé pour la préparation de petites vésicules unilamellaires d'un diamètre inférieur à 200 nm (2000 Å) appropriées pour les applications biologiques, dans lequel:
   (a) on hydrate des molécules amphiphiles qui sont des phospholipides ou des phosphoglycérides ayant des chaînes hydrocarbonées d'au moins 16 atomes de carbone, et éventuellement d'autres composants capables de former des vésicules lipidiques qui sont stables in vivo;
   (b) on disperse ledit lipide hydraté dans un appareil d'homogénéisation ayant un moyen de recirculation à une pression de 55 à 90 MPa(8.000 à 13.000 p.s.i) et à une température de 50°C à 80°C pour générer une microémulsion contenant de petits vésicules lipidiques unilamellaires de moins de 200 nm(2000 Å); et
   (c) on sépare lesdites petites vésicules unilamellaires de toute matière éventuellement non-encapsulée.

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2. Procédé selon la revendication 1 dans lequel les molécules amphiphiles et autres composants capables de former des vésicules lipidiques comprennent un phospholipide et du cholestérol dans un rapport molaire de 2 à 1.

3. Procédé selon la revendication 1 ou 2 dans lequel la concentration finale de lipide total dans la microémulsion est de 10 mg/ml.

4. Procédé selon la revendication 1 dans lequel l'étape (a) comprend:
   (a) (i) la dissolution desdites molécules amphiphiles et d'autres composants capables de former des vésicules lipidiques dans une solution de solvant organique;
   (a) (ii) la dessication de ladite solution en une pellicule lipidique; et
   (a) (iii) la réhydratation de la pellicule lipidique séchée.

5. Procédé selon la revendication 4 pour la préparation de vésicules appropriées pour une utilisation dans le ciblage des tumeurs corporelles aux fins de localisation et de diagnostic des tumeurs, dans lequel: dans l'étape (a) (i), on dissout les molécules amphiphiles et au moins un autre composant, y compris un ionophore;
   dans l'étape (a) (iii), on réhydrate la pellicule lipidique séchée avec une solution physiologique de sel tamponnée auphosphate contenant un agent de chélation faible; dans l'étape (b), on disperse ledit lipide réhydraté pour générer une microémulsion contenant des vésicules lipidiques avec l'ionophore incorporé dans la bicouche lipidique et lesdites vésicules contenant l'agent de chélation;
   et, après l'étape (c),
   (d) on charge lesdites vésicules avec un cation radioactif pour détecter la localisation desdites vésicules lorsqu'on les administre dans un corps.

6. Procédé selon la revendication 4 pour la préparation de vésicules pour utilisation dans le traitement d'une tumeur corporelle, dans lequel :
   dans l'étape (a) (iii), on réhydrate la pellicule lipidique séchée avec une solution contenant un agent thérapeutique; et
   dans l'étape (b) on disperse ledit lipide hydraté pour générer une microémulsion contenant des vésicules lipidiques contenant l'agent thérapeutique.

7. Procédé selon la revendication 4, 5 ou 6 dans lequel ledit solvant organique est un éther, le chloroforme ou un alcool.

8. Procédé selon l'une quelconque des revendications precédentes dans lequel on conduit ladite étape de dispersion dans un appareil d'homogénéisation dans lequel ladite solution lipidique peut être maintenue dans un intervalle de température séléctionné.

9. Procédé selon la revendication 8 dans lequel ledit appareil comprend un réservoir d'échange de chaleur.

10. Procédé selon l'une quelconque des revendications précédentes dans lequel on conduit ladite étape de dispersion en faisant fonctionner ledit appareil d'homogénéisation à une pression de 55 à 83 MPa (8.000 à 12.000 p.s.i) et à une température de solution maintenue entre 50° et 80° C pendant une durée allant de 15 à 90 minutes.

11. Procédé selon l'une quelconque des revendications 1 à 9 dans lequel on conduit ladite étape de dispersion en faisant fonctionner ledit appareil d'homogénéisation à une pression de 55 à 69 MPa (8.000 à 10.000 p.s.i) et à une température de réservoir de 70-75° C pendant environ 60 minutes.

12. Procédé selon l'une quelconque des revendications 1 à 9 dans lequel on conduit ladite étape de dispersion en faisant fonctionner l'appareil d'homogénéisation à une pression de 69 à 83 MPa (10.000 à 12.000 p.s.i) et à une température de réservoir de 50-55° C pendant environ 60 minutes.

13. Procédè selon l'une quelconque des revendications 1 à 9 dans lequel on conduit ladite étape de dispersion en faisant fonctionner ledit appareil d'homogénéisation à une pression d'environ 69 MPa (10.000 p.s.i) et à une température de réservoir de 70°-75° C pendant environ 60 minutes.

14. Procédé selon la revendication 1 ou 6 dans lequel lesdites molécules amphiphiles sont des phospholipides qui sont la phosphatidylcholine, la phosphatidyléthanolamine ou la phosphatidylsérine.

15. Procédé selon la revendication 1 ou 5 dans lequel le phospholipide est la distéaroyl-phosphatidylcholine et/ou la dipalmitoyl-phosphatidylcholine.

16. Procédé selon la revendication 15 lorsqu'elle se rattache à la revendication 5 dans lequel lesdites molécules amphiphiles sont des phospholipides et lesdits autres composants capables de former des vésicules comprennent du cholestérol et un ionophore.

17. Procédé selon la revendication 16 dans lequel la composition des vésicules comprend un phospholipide, du cholestérol et un ionophore dans un rapport molaire de 1:2:0,004.

18. Procédé selon la revendication 16 ou 17 dans lequel l'ionophore est le A23187.

19. Procédé selon l'une quelconque des revendications 5, 16, 17 ou 18 dans lequel l'agent de chélation est l'acide nitriloacétique.

20. Procédé selon l'une quelconque des revendications 5, 16, 17, 18 ou 19 dans lequel le cation radioactif est $^{111}In^{3+}$.

21. Procédé selon l'une quelconque des revendications 5 ou 16 à 20 dans lequel la concentration finale de lipide total dans la microémulsion est de 25 mg/ml.

22. Procédé selon la revendication 6 dans lequel le phospholipide est la distéaroyl-phosphatidylcholine.

23. Procédé selon la revendication 6 ou 22 dans lequel ladite étape de dissolution de substances lipidiques dans un solvant organique comprend en outre l'addition de cholestérol.

24. Procédé selon l'une quelconque des revendications 6, 22 ou 23 dans lequel la composition des vésicules comprend un phospholipide, du cholestérol et un agent thérapeutique, ledit agent thérapeutique étant contenu dans la solution utilisée pour hydrater les molécules amphiphiles.

25. Procédé selon l'une quelconque des revendications 6 ou 22 à 24 dans lequel ledit agent thérapeutique est un agent chimiothérapeutique.

26. Procédé selon la revendication 25 dans lequel ledit agent chimiothérapeutique est un antibiotique qui comprend la Daunomycine, la Bléomycine, l'Adriamycine, l'Actinomycine D, la Mytomycine C ou la Mithramycine.

27. Procédé selon la revendication 25 dans lequel ledit agent chimiothérapeutique est un agent alcoylant comprenant le chlorambucil, le cyclophosphamide ou la triéthylènemélamine.

28. Procédé selon la revendication 25 dans lequel ledit agent chimiothérapeutique est un antimétabolite comprenant le méthotrexate, le fluoro-5-uracile, la mercapto-6-purine ou l'arabinosylcytosine.

29. Procédé selon la revendication 25 dans lequel ledit agent chimiothérapeutique est le méthotrexate.

30. Procédé selon l'une quelconque des revendications 6 ou 22 à 24 dans lequel ledit agent thérapeutique est un agent radiothérapeutique.

31. Procédé selon la revendication 30 dans lequel ledit agent radiothérapeutique est un radionuclide qui comprend l'iode 131, l'yttrium 90 ou le phosphore 32.

Ansprüche

1. verfahren zur Herstellung kleiner, unilamellarer Vesikel mit einem Durchmesser von weniger als 200 nm (2000 Å), die für biologische Applikationen geeignet sind, umfassend:
(a) Hydratisieren amphiphiler Moleküle, die Phospholipide oder Phosphoglyceride, die Kohlenwasserstoffketten mit mindestens 16 Kohlenstoffatomen besitzen, sind, und gegebenenfalls andere

Komponenten, die zur Bildung flüssiger Vesikel, die in vivo stabil sind, fähig sind;
(b) Dispergieren des hydratisierten Lipids in einem Homogenisierapparat mit Rückführungseinrichtungen bei einem Druck von 55 bis 90 MPa (8000 bis 13000 p.s.i.) und einer Temperatur von 50° C bis 80° C, um eine Mikroemulsion zu erzeugen, die kleine unilamellare Lipidvesikel von weniger als 200 nm (2000 Å) enthält; und
(c) Abtrennung der kleinen, unilamellaren Vesikel von irgendwelchen nicht eingekapselten Materialien.

2. Verfahren nach Anspruch 1, dadurch gekennzeichnet, daß die amphiphilen Moleküle und anderen zur Ausbildung von Lipidvesikeln fähigen komponenten ein Phospholipid und Cholesterin in einem Molverhältnis von 2 zu 1 umfassen.

3. Verfahren nach Anspruch 1 oder 2, dadurch gekennzeichnet, daß die Endkonzentration des Gesamtlipids in der Mikroemulsion 10 mg/ml ist.

4. Verfahren nach Anspruch 1, dadurch gekennzeichnet, daß die Stufe (a) umfaßt:
   (a) (i) Auflösen der amphiphilen Moleküle und anderer zur Ausbildung von Lipidvesikeln fähiger Komponenten in einer Lösung eines organischen Lösungsmittels;
   (a) (ii) Trocknen der Lösung zu einem Lipidfilm; und
   (a) (iii) Rehydratisierung des getrockneten Lipidfilms.

5. Verfahren nach Anspruch 4 zur Herstellung von Vesikeln, die zur Verwendung zur Festellung von Tumoren in einem körper zur Lokalisierung und Diagnose der Tumore geeignet sind, umfassend:
in Stufe (a) (i) die Auflösung der amphiphilen Moleküle und mindestens einer anderen Komponente, einschließlich eines Ionophors;
in Stufe (a) (iii) die Rehydratisierung des getrockneten Lipidfilms mit Phosphat-gepufferter Salzlösung, die ein schwaches Gelatisierungsmittel enthält;
in Stufe (b) die Dispersion dieses rehydratisierten Lipids um eine Mikroemulsion zu bilden, die Lipidvesikel mit dem in die Lipiddoppelschicht eingebauten Ionophor enthält und diese Vesikel das Gelatisierungsmittel enthalten;
und der Stufe (c) folgend,
   (d) Beschicken dieser Vesikel mit einem radioaktiven Kation zur Bestimmung der Lokalisierung dieser Vesikel, wenn sie in einen Körper verabreicht wurden.

6. Verfahren nach Anspruch 4 zur Herstellung von Vesikeln zur Verwendung bei der Behandlung eines Tumors in einem körper umfassend:
in Stufe (a) (iii) die Rehydratisierung des getrockneten Lipidfilms mit einer Lösung, die ein therapeutisches Mittel enthält; und
in Stufe (b) die Dispersion dieses hydratisierten Lipids um eine Mikroemulsion zu bilden, die Lipidvesikel enthält, die das therapeutische Mittel enthalten.

7. Verfahren nach Anspruch 4, 5 oder 6, dadurch gekennzeichnet, daß das organische Lösungsmittel ein Ether, Chloroform oder Alkohol ist.

8. Verfahren nach einem der vorhergehenden Ansprüche, dadurch gekennzeichnet, daß die Dispersionsstufe durchgeführt wird in einem Homogenisierungsapparat, in dem die Lipidlösung in einem ausgewählten Temperaturbereich gehalten werden kann.

9. Verfahren nach Anspruch 8, dadurch gekennzeichnet, daß der Apparat ein Wärmeaustauschreservoir umfaßt.

10. Verfahren nach einem der vorhergehenden Ansprüche, dadurch gekennzeichnet, daß die Dispergierstufe durchführt, indem man den Homogenisierapparat bei einem Druck von 55 bis 83 MPa (8000 bis 12000 p.s.i.) und einer bei 50° bis 70° C gehaltenen Lösungstemperatur während einer Zeit von 15 bis 90 Minuten betreibt.

11. Verfahren nach einem der Ansprüche 1 bis 9, dadurch gekennzeichnet, daß die Dispergierstufe durchgeführt wird indem man den Homogenisierapparat bei einem Druck von 55 bis 69 MPa (8000 bis

10000 p.s.i.) und einer Reservoirtemperatur von 70° bis 75° C während ca. 60 Minuten betreibt.

12. verfahren nach einem der Ansprüche 1 bis 9, dadurch gekennzeichnet, daß man die Dispergierstufe durchführt, indem man den Homogenisierapparat bei einem Druck von 69 bis 83 MPa (10000 bis 12000 p.s.i.) und einer Reservoirtemperatur von 50° bis 55° C während ca. 60 Minuten betreibt.

13. Verfahren nach einem der Ansprüche 1 bis 9, dadurch gekennzeichnet, daß man die Dispergierstufe durchführt, indem man den Homogenisierapparat bei einem Druck von ca. 69 MPa (10000 p.s.i.) und einer Reservoirtemperatur von 70° bis 75° C während ca. 60 Minuten betreibt.

14. Verfahren nach Anspruch 1 oder 6, dadurch gekennzeichnet, daß die amphiphilen Moleküle Phospholipide sind, nämlich Phosphatidylcholin, Phosphatidylethanolamin oder Phosphatidylserin.

15. Verfahren nach Anspruch 1 oder 5, dadurch gekennzeichnet, daß das Phospholipid Distearoylphosphatidylcholin und/oder Dipalmitoylphosphatidylcholin ist.

16. Verfahren nach Anspruch 15, wenn abhängig von Anspruch 5, dadurch gekennzeichnet, daß die amphiphilen Moleküle Phospholipide sind und die anderen zur Vesikelbildung fähigen Komponenten Cholesterin und ein lonophor umfassen.

17. Verfahren nach Anspruch 16, dadurch gekennzeichnet, daß die Zusammensetzung der Vesikel Phospholipid, Cholesterin und lonophor in einem Molverhältnis 1:2:0.004 umfaßt.

18. Verfahren nach Anspruch 16 oder 17, dadurch gekennzeichnet, daß das lonophor A23187 ist.

19. Verfahren nach einem der Ansprüche 5, 16, 17 oder 18, dadurch gekennzeichnet, daß das Gelatisiermittel Nitriloessigsäure ist.

20. Verfahren nach einem der Ansprüche 5, 16, 17, 18 oder 19, dadurch gekennzeichnet, daß das radioaktive Kation $^{111}In^{3+}$ ist.

21. Verfahren nach einem der Ansprüche 5 oder 16 bis 20, dadurch gekennzeichnet, daß die Endkonzentration des Gesamtlipids in der Mikroemulsion 25 mg/ml ist.

22. Verfahren nach Anspruch 6, dadurch gekennzeichnet, daß das Phospholipid Distearoylphosphatidylcholin ist.

23. Verfahren nach Anspruch 6 oder 22, dadurch gekennzeichnet, daß die Stufe des Auflösens der Lipidsubstanzen in einem organischen Lösungsmittel außerdem die Zugabe von Cholesterin umfaßt.

24. Verfahren nach einem der Ansprüche 6, 22 oder 23, dadurch gekennzeichnet, daß die Zusammensetzung der Vesikel Phospholipid, Cholesterin und ein therapeutisches Mittel umfaßt, wobei das therapeutische Mittel in der zu Hydratisierung der amphiphilen Moleküle verwendeten Lösung enthalten ist.

25. Verfahren nach einem der Ansprüche 6 oder 22 bis 2, dadurch gekennzeichnet, daß das therapeutische Mittel ein chemotherapeutisches Mittel ist.

26. Verfahren nach Anspruch 25, dadurch gekennzeichnet, daß das chemotherapeutische Mittel ein Antibiotikum ist, das Daunomycin, Belomycin, Adriamycin, Actinomycin D, Mytomycin C oder Mithramycin umfaßt.

27. Verfahren nach Anspruch 25, dadurch gekennzeichnet, daß das chemotherapeutische Mittel ein Alkylierungsmittel ist umfassend Chlorambucil, Cyclophosphamid oder Triethylenmelamin.

28. Verfahren nach Anspruch 25, dadurch gekennzeichnet, daß das therapeutische Mittel ein Antimetabolit ist umfassend Methotrexat, 5-Fluorouracil, 6-Mercaptopurin oder Arabinosylcytosin.

29. Verfahren nach Anspruch 25, dadurch gekennzeichnet, daß das chemotherapeutische Mittel Methotrexat

ist.

30. Verfahren nach einem der Ansprüche 6 oder 22 bis 24, dadurch gekennzeichnet, daß das therapeutische Mittel ein radiotherapeutisches Mittel ist.

31. Verfahren nach Anspruch 30, dadurch gekennzeichnet, daß das radiotherapeutische Mittel ein Radionuclid ist, das Iod 131, Yttrium 90 oder Phosphat 32 umfaßt.

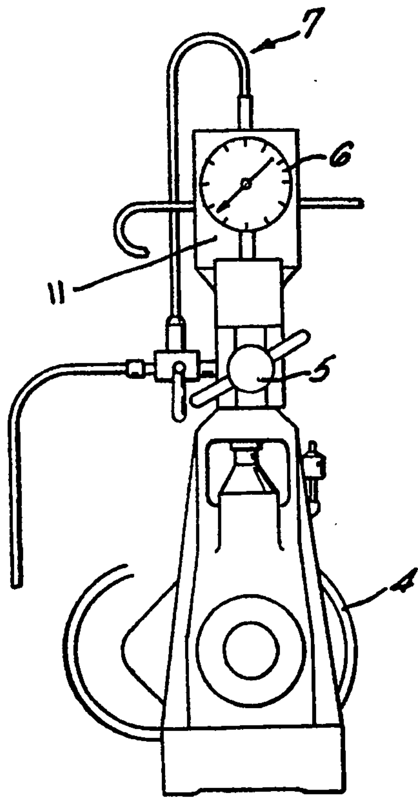

FIG. 1

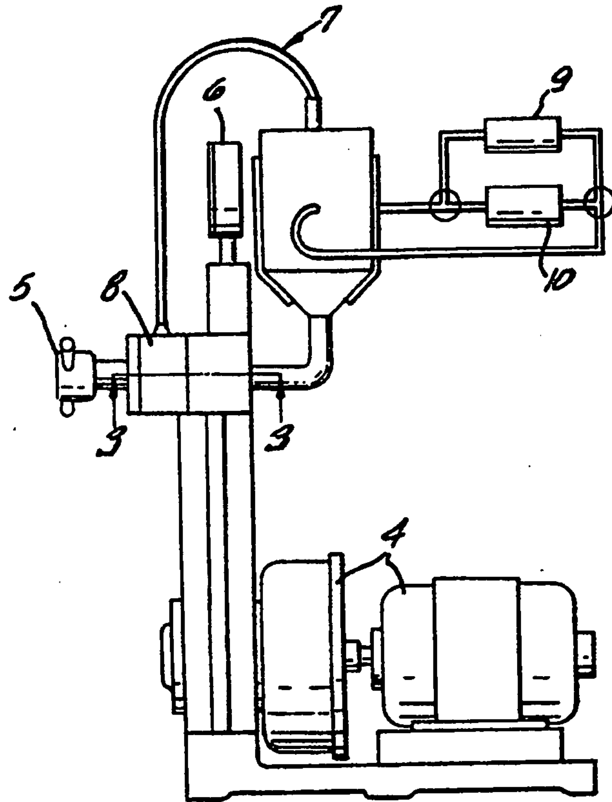

FIG. 2

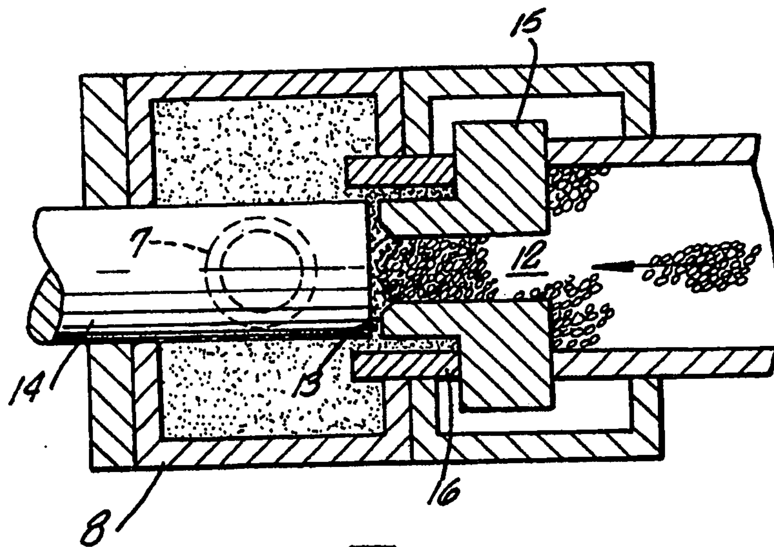

FIG. 3